

Cytochrome P450 CYP3A5 in the human anterior pituitary gland

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Abstract The cytochromes P450 are a key group of enzymes involved in the metabolism of xenobiotics and several biologically active endogenous compounds. The expression of CYP3A5 has been identified by reverse transcriptase-polymerase chain reaction in human pituitary gland and shown by immunohistochemistry to be localized to growth hormone containing cells of the anterior pituitary gland. This is the first direct identification of an individual P450 subfamily in the pituitary gland and the presence of CYP3A in the pituitary gland may play a role in regulating growth hormone secretion.

Key words: Cytochrome P450; Pituitary gland; Somatotroph

1. Introduction

The P450s are a multi-gene family of constitutive and inducible enzymes which play a central role in the oxidative metabolism of a diverse range of xenobiotics and a variety of biologically active endogenous compounds [1–4]. In general terms there are two broad classes of mammalian P450s: a large class of P450s which are primarily involved in xenobiotic metabolism and a second smaller group specifically involved in steroid hormone synthesis. The xenobiotic metabolizing P450 families (CYP1, CYP2 and CYP3) are expressed mainly in tissues exposed to environmental and dietary chemicals [5–7]. They may, however, also have a role in cell regulation in view of their involvement in the metabolism of physiological chemicals active in inter- and intra-cellular signaling, including steroid hormones, eicosanoids and fatty acids [8].

The pituitary gland has a central role in homeostasis and there is evidence in the rat that the pituitary contains P450 [9–11], the activity of which contributes to the regulation of pituitary polypeptide hormone secretion [11–14].

In this study we have identified the cell-specific expression of CYP3A5 in growth hormone containing cells of the human anterior pituitary gland.

2. Materials and methods

2.1. Tissue

Twelve pituitary glands (5 males, 7 females, age range 62–84 years) were obtained, with permission, from autopsies performed in the Department of Pathology, University of Aberdeen, within 24 h of death. None of the patients had any evidence of pituitary disease, other endo-

crine disease or degenerative neurological disease. Each pituitary gland was divided into two portions: one part was frozen in liquid nitrogen and used for RT-PCR and the other part was fixed in 10% neutral-buffered formalin for 24 h, then embedded in wax and used for immunohistochemistry.

2.2. RT-PCR

Total RNA was extracted using essentially the method of Chomczynski and Sacchi [15]. The recovered RNA pellet was resuspended in 100 μ l distilled water pretreated with 0.1% (v/v) DEPC (Sigma, Poole, UK). Single stranded cDNA was synthesised from the RNA using oligo (dT)₁₅ (Promega, Southampton, UK) with the following reaction conditions: RNA (20 μ l), 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, 10 mM dithiothreitol (Sigma), 40 units human placental ribonuclease inhibitor (Promega), 0.125 mM each of dATP, dCTP, dGTP and dTTP (Promega), 18 units avian myeloblastosis virus reverse transcriptase (Promega) and 0.5 mg oligo (dT)₁₅ primer (Promega) in 50 mM Tris-HCl, pH 8.3. cDNA was then synthesised at 42°C for 60 min and the synthesis was stopped by heating the reaction mixture to 80°C for 5 min.

CYP3A primers were designed to recognise sequences common to CYP3A4, CYP3A5 and CYP3A7 and to give a product of 714 bp in each case [16–18]. The specific sequences are located on exon 7 and exon 11 of CYP3A4 [19]. The primers were synthesised and purified with HPLC by Oswel DNA Service (Edinburgh, UK) with the following sequences:

Forward primer 5'-GGC CTA CAG CAT GGA TGT GAT CAC-3' (CYP3A4, nt 621–644; CYP3A5, nt 618–641; CYP3A7, nt 534–557)
Reverse primer 5'-GAG GAA CTT CTC AGG CTC TGT CCA G-3' (CYP3A4, nt 1311–1335; CYP3A5, nt 1308–1332; CYP3A7, nt 1224–1248)

PCR using the hot start technique was carried out in a 100 μ l reaction volume consisting of 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.4 mM each of dATP, dCTP, dGTP and dTTP, 0.5 U Taq DNA polymerase (Promega), 2 μ l cDNA and 50 pmol of each primer. 30 cycles of amplification were performed using the following conditions: 94°C for 1 min (denaturation); 53°C for 0.5 min (annealing); 72°C for 1 min (extension); and a final further 2 min extension at 72°C. Both positive and negative controls were included in the PCR. The positive control was a 2.3 kb CYP3A4 cDNA (isolated from a human liver cDNA library in our laboratories) which had been cloned into λ gt11. The negative control was DEPC treated water in place of template cDNA. After PCR 5 μ l of the PCR product was electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. The PCR product was purified with a Wizard miniprep DNA purification system (Promega) used according to the manufacturers instructions and analysed by Southern hybridization and direct sequencing. Southern hybridization was carried out according to Sambrook et al. [20] using a probe generated by PCR from the CYP3A4 plasmid as described above. The probe was labeled with ³²P by nick translation using a kit supplied by Amersham International (Amersham, UK). PCR products were sequenced using a modification of the direct dideoxy sequencing technique of Sanger et al. [21] with a T7 sequencing kit (Pharmacia, Milton Keynes, UK) used according to the manufacturers protocol with dimethyl sulfoxide used as denaturant.

2.3. Antibodies

CYP3A was identified with monoclonal antibody HP3 which recognises all known forms of human hepatic CYP3A [22]. Rabbit polyclonal

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Abbreviations: EPC, diethyl pyrocarbonate; P450, cytochrome P450; CYP 3A5, the 5th member of the cytochrome P450 family 3A; FITC, fluorescein isothiocyanate; RT-PCR, reverse transcriptase polymerase chain reaction; TBS, 0.05 M Tris-HCl-buffered saline pH 7.6.

antibodies to the different anterior pituitary hormones (adrenocorticotrophic hormone, follicle stimulating hormone, growth hormone, luteinising hormone, prolactin and thyroid stimulating hormone) were obtained from the National Institutes of Health, Bethesda USA. Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin, horseradish peroxidase conjugated swine anti-rabbit and FITC conjugated swine anti-rabbit immunoglobulins were purchased from Dako Ltd, High Wycombe, UK.

2.4. Immunohistochemistry

Immunohistochemistry was performed on sections of formalin fixed wax embedded pituitary gland using an immunoperoxidase technique as previously described [23]. Formalin fixed wax embedded sections of normal liver were used as a positive control [24]. Briefly, endogenous peroxidase was blocked and the sections were then sequentially incubated with the different primary antibodies followed by the relevant peroxidase conjugated second layer antibody. After each antibody incubation excess unbound antibody was removed by washing with TBS, and sites of antibody binding were identified using 3,3'-diaminobenzidine (Sigma) as the peroxidase substrate.

CYP3A and pituitary hormones were co-localized by double staining on the same section using immunofluorescence for antibodies to anterior pituitary hormones and immunoperoxidase with monoclonal antibody HP3 for CYP3A. A second layer FITC conjugated swine anti-rabbit immunoglobulin (1/10 in TBS, Dako) was used for the immunofluorescence detection of anterior pituitary hormone immunoreactivity. Double labeled sections were examined with transmitted light and epi-fluorescence microscopy (Nikon, London, UK) with a filter block (B2, Nikon) for FITC (excitation 450–490 nm, dichroic mirror 510 nm and emission filter 520 nm).

3. Results

3.1. RT-PCR

The RNA isolated from each pituitary gland displayed minimal evidence of degradation as judged by agarose gel electrophoresis following denaturation by formaldehyde (data not shown). Analysis of the PCR product by visual inspection of an ethidium bromide stained agarose gel showed a clear single band of the predicted size (714bp) in 6 of the 12 pituitary samples (Fig. 1). Southern transfer and hybridization, however, showed the presence of an amplification product of the correct size in all 12 samples (Fig. 2). Sequencing of the individual PCR products showed identity with CYP3A5.

3.2. Immunohistochemistry

All 12 pituitary glands demonstrated positive immunoreactivity for CYP3A using monoclonal antibody HP3, with strong cytoplasmic (i.e. non-nuclear) immunostaining of a discrete and major population of cells within the anterior pituitary gland. The posterior pituitary gland did not immunostain for CYP3A. In the pituitary gland the distribution of CYP3A immunostaining with HP3 was similar to the pattern of immunoreactivity

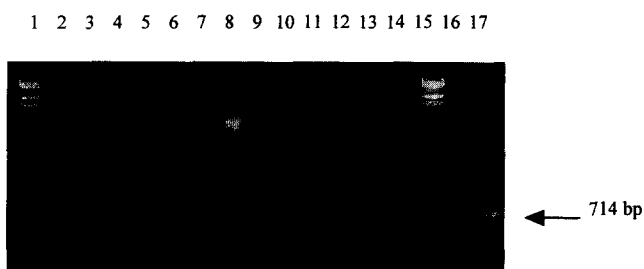


Fig. 1. Ethidium bromide stained agarose gel of pituitary PCR products. Lanes 1, 8 and 15 molecular weight markers, lanes 2–7 pituitary samples, lanes 9–14 pituitary samples, lane 16 negative control (DEPC-treated water), lane 17 positive control (CYP3A4 plasmid).

observed for growth hormone. Double immuno-labelling of the pituitary sections for both CYP3A and growth hormone confirmed that CYP3A and growth hormone were co-localized to the same cells (Fig. 3).

4. Discussion

In this study we have identified the expression of P450 CYP3A5 within the human pituitary gland and localized its immunoreactivity to growth hormone containing cells of the anterior pituitary. Although there is circumstantial evidence for P450 in rat pituitary, namely a carbon monoxide-binding spectrum and characteristic monooxygenase activities [9–11], this is the first direct identification of an individual P450 subfamily in pituitary, and the first evidence for P450 expression in human pituitary. The human CYP3A gene subfamily of xenobiotic metabolising P450s contains three known forms, CYP3A4, CYP3A5 and CYP3A7; are capable of metabolising a wide range of xenobiotics and endogenous compounds and are expressed in both hepatic and extra-hepatic tissues [6,25]. CYP3A5 although only expressed in about 25% of livers has been shown to be much more frequently expressed in extra-hepatic tissues and indeed has been shown to be the major form of CYP3A expressed in kidney [26,27].

The strategy we have adopted to identify the CYP3A expressed in human pituitary has been to perform RT-PCR using primers which recognise sequences common to all known human CYP3A forms and then to sequence the PCR product. A sequence identical to CYP3A5 was obtained from each PCR product. Due to the small size of the pituitary gland, it was not possible to confirm the molecular weight of the CYP3A-immunoreactive pituitary protein by immunoblotting.

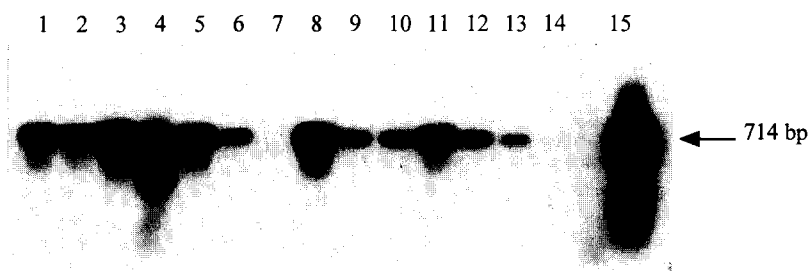
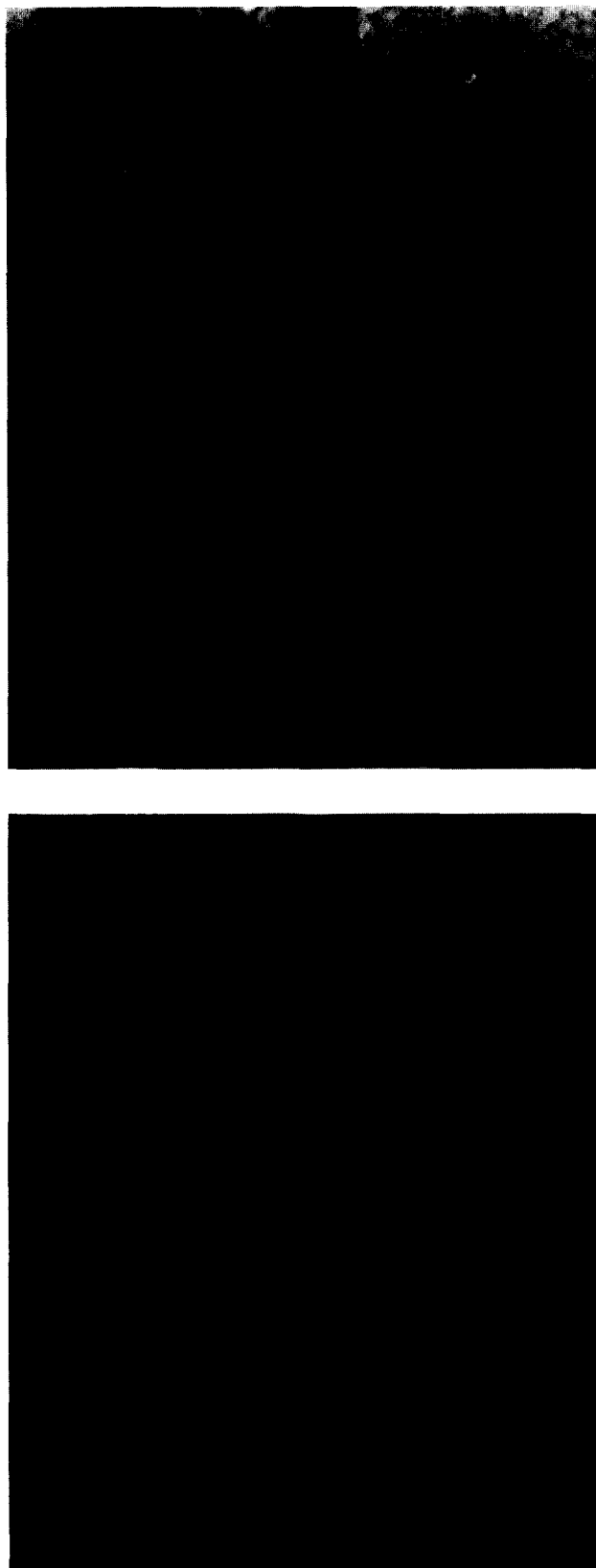


Fig. 2. Autoradiogram of CYP3A PCR products following Southern transfer and hybridization. Lanes 1–6 pituitary samples, lanes 7 and 14 negative controls (DEPC-treated water), lanes 8–13 pituitary samples, lane 15 positive control (CYP3A4 plasmid). The individual pituitary samples are in the same consecutive order as in Fig. 1.



CYP3A was localized specifically to growth hormone containing cells of the anterior pituitary gland. Here it may play a role in regulating growth hormone secretion, since the release

Fig. 3. Double immunolabelling of anterior pituitary gland for CYP3A and growth hormone. (a) CYP3A immunoreactivity with monoclonal antibody HP3 using immunoperoxidase staining. (b) Growth hormone immunoreactivity with a polyclonal antibody using immunofluorescence staining. The same section is viewed by (a) bright field microscopy and (b) epi-fluorescence microscopy to show that both CYP3A and growth hormone are co-localized in anterior pituitary cells. (To facilitate the comparison arrows indicate two cells which are the same in both photographs). Magnification for (a) and (b) $\times 1000$.

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of growth hormone from rat pituitary somatotrophs in vitro is inhibited by broad spectrum cytochrome P450 inhibitors [14] and stimulated by epoxyeicosatrienoic acid metabolites of arachidonic acid produced by P450 [12,13]. Moreover, these metabolites can also be metabolically inactivated by P450 [4]. The presence of a steroid-hydroxylating form of P450 in the pituitary gland also provides a potential mechanism for limiting the steroid hormone feedback regulation of growth hormone secretion [28].

In conclusion, the concerted use of RT-PCR and immunohistochemistry has identified and localized cytochrome P450 CYP3A5 specifically to the growth hormone containing cells of the human anterior pituitary gland.

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